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# Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics

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**Abstract**

Liposomes were prepared by stepwise extrusion through 5, 1, 0.4, 0.2, 0.1 and 0.05 μm pore sizes using two different filter-extruders, the continuous high pressure device Dispex Maximator® (CE) or alternatively the discontinuous Avestin LiposoFast™ (DE). The liposome dispersions obtained were compared in terms of particle size, lamellarity and encapsulation efficiency of calcein. The liposomes were smaller with CE than DE at all stages due to higher flow rates and pressure drops, except for final filter pore size (0.05 μm) where both preparations had similar sizes. The particle size analysis technique itself had a strong influence on the liposome sizes measured. For bigger liposomes (extruded through 0.4 μm filters) the Nicomp 370 revealed bigger volume-based mean particle sizes along with more stringent differences between volume-based and number-based diameters than the Malvern Zetasizer. In contrast, for small liposomes extruded through 0.05 μm filters, similar liposome sizes were found no matter which of the two PCS techniques or cryo-transmission electron microscopy was used. In congruence to the liposome sizes measured, encapsulation efficiencies were smaller for CE than DE at all filter stages except the final (0.05 μm). No lipid loss occurred and lyso-phosphatidylcholine formation was negligible irrespective of which extrusion technique was used. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Liposome; Vesicle; Particle size; Filter; Extrusion; Phosphatidylcholine

## 1. Introduction

Extrusion of hydrated lipid films is a common method for production of liposomes on a laboratory scale and there are numerous reports on liposome preparation with various devices such as: Lipex extruder (Nayar et al., 1989; Schubert et al., 1991; Tilcock et al., 1992; Elorza et al., 1993; Hope et al., 1993), Nuclepore 24 mm filter holder (Olson et al., 1979; Szoka et al., 1980), Millipore high pressure filter holder (Chapman et al., 1991), Schleicher and Schuell ultrafiltration device (Am-

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selem et al., 1989–90), Hamilton syringes as mini-extruder (MacDonald et al., 1991) and a high pressure plexiglas filtration cell (Turanek, 1994). In a previous study a novel device for preparation of liposomes by filter extrusion has been introduced which differs from earlier devices by its ability to work continuously and at high pressures (Sachse and Rössling, 1994; Schneider et al., 1994). Its ability to produce contrast-carrying liposomes in an efficient and GMP-compliant manner has been demonstrated (Schneider et al., 1995, 1996; Sachse, 1998). Besides the wide choice of different extrusion devices used for liposome preparation also the process parameters have been varied, e.g. filter pore sizes, number of passages and inclusion of freeze-thaw cycles as part of the production protocol: (Hope et al., 1985; Mayer et al., 1986; Jousma et al., 1987; Nayar et al., 1989; Chapman et al., 1991; MacDonald et al., 1991; Schubert et al., 1991; Tilcock et al., 1992; Elorza et al., 1993; Schneider et al., 1994, 1995; Turanek, 1994). Another type of variation concerned the employed lipid compositions and lipid concentrations. Lipid concentrations used ranged from $<10$ mg/ml (Olson et al., 1979; Szoka et al., 1980) over 20–25 mg/ml (Jousma et al., 1987; Lesieur et al., 1991; MacDonald et al., 1991), 40–50 mg/ml (Hope et al., 1985; Nayar et al., 1989) and 100 mg/ml (Mayer et al., 1986) to $>150$ mg/ml (Turanek, 1994; Schneider et al., 1995). Furthermore encapsulation of various drugs was described: cytosine arabinoside (Olson et al., 1979; Szoka et al., 1980), doxorubicin (Amselem et al., 1989–90), sucrose (Olson et al., 1979; Turanek, 1994), inulin (Nayar et al., 1989; Schubert et al., 1991), iopromide (Schneider et al., 1995), calcein (MacDonald et al., 1991; Grit and Crommelin, 1992) and carboxyfluorescein (Jousma et al., 1987; Elorza et al., 1993).

In addition different analytical methods were used to characterise the resulting liposomes: size analysis by freeze-fracture electron microscopy (Olson et al., 1979; Mayer et al., 1986; Jousma et al., 1987; Nayar et al., 1989), negative staining electron microscopy (Olson et al., 1979; Szoka et al., 1980; Turanek, 1994; Schneider et al., 1995), gel exclusion chromatography (Lesieur et al., 1991) or photon correlation spectroscopy by means of different apparatus like Malvern (Grit and Crommelin, 1992), Nicomp (Mayer et al., 1986; Nayar et al., 1989; Tilcock et al., 1992; Schneider et al., 1995) or Coulter (Amselem et al., 1989–90; Lesieur et al., 1991; Elorza et al., 1993).

There is thus plenty of data available on liposome preparation by filter extrusion. But, it is unclear if the literature results can be used for direct comparison because it is not known whether the observed differences in the liposome characteristics are due to the use of different extrusion devices, different process parameters, or differences in the employed analytical techniques. The aim of the present study was to prepare liposomes under standardised conditions by two different extrusion devices: a discontinuous device (Avestin Liposo-Fast™-50) and a continuous high-pressure extruder (Dispex Maximator® model: HPE 12.0–100) and to characterise the resulting liposomes by standardised analytical techniques in terms of particle size, encapsulation efficiency (EE), lamellarity ($^{31}$P-NMR) and lipid content. Thus, by holding both, preparative and analytical process parameters constant, the influence of the extrusion device should be studied. Furthermore, for certain liposome preparations particle size analysis was carried out in parallel in different ways, cryo-electron microscopy and PCS using a Nicomp Model 370 and a Malvern Zetasizer 3000 in order to evaluate the influence of the size analysis method.

## 2. Materials and methods

### *2.1. Starting materials*

Soy phosphatidylcholine (SPC, Lipoid S100) and soy phosphatidylglycerol (SPG, Lipoid SPG) were purchased from Lipoid KG, Ludwigshafen, Germany. Cholesterol (CH) was obtained from Solvay Duphar B.K., Veenendal. The Netherlands. Calcein was obtained from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany.

## 2.2. Liposome preparation

A thin lipid film was formed by dissolving the lipid mixture (SPC:CH:SPG 6.25:3:0.75 molar ratio = standard) in ethanol (96% reagent grade, E. Merck, Darmstadt, Germany) and subsequent removal of the solvent by rotary evaporation at 60 °C to complete dryness. The lipid composition was chosen with respect to extrudability and stability of the liposome dispersions, for further details see Schneider (Schneider et al., 1995). After hydration of the lipid film in 20 mM isotonic phosphate buffer pH 6.5 containing 5 mM calcein and 0.013% EDTA the resulting multilamellar vesicles (MLV) were frozen (methanol/dry ice, − 70 °C) and thawed (water-bath, 70 °C) three times. This vesicle suspension was divided into two equal parts which were then extruded five times through double-stacked membranes (polycarbonate (PC), Nuclepore®, Costar GmbH, Bodenheim, Germany) with decreasing pore sizes (5.0, 1.0, 0.4, 0.2, 0.1 and 0.05 μm) either with the continuous high pressure extruder (CE; Maximator® model: HPE 12.0-100, Dispex, Berlin, Germany) or the discontinuous extruder (DE; Liposo-Fast™-50, Avestin Inc., Ottawa, Canada) (see schematic drawings Fig. 1a and b). For comparability reasons the continuous extruder also was run in a discontinuous mode. For DE an additional support membrane the so-called 'drain disk' (polypropylene 45 μm pore size, Millipore GmbH, Eschborn, Germany) was needed. The final liposomes were stored in sterile 10-ml vials after filtration through 0.2 μm cellulose acetate membranes (Sartorius, Göttingen, Germany). The total lipid concentration was always 150 mg/ml.

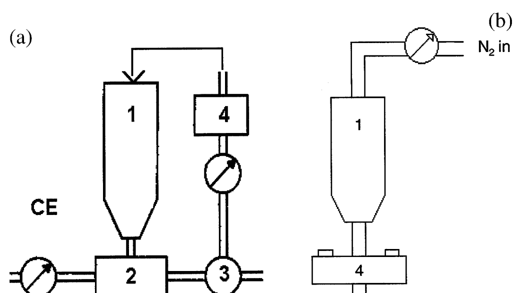


Fig. 1. (a) Continuous extruder (CE); and (b) discontinuous extruder (DE); 1 = supply chamber, 2 = pump, 3 = three-way-stopcock, 4 = filter holder.

## 2.3. Liposome size determination

### 2.3.1. PCS (Nicomp)

Normally liposome size was determined by photon correlation spectroscopy using a Submicron Particle-Sizer Autodilute®, Model 370, Nicomp Instr. Corp., Santa Barbara, CA, USA. The average vesicle size distribution was determined either by volume-based or by number-based gaussian or so-called 'Nicomp' (non-gaussian) fit to raw data which had been collected over 5 min at 23 °C at an angle of 90°.

### 2.3.2. PCS (Malvern)

For comparison selected liposome dispersions were analysed in parallel using a Malvern Zetasizer 3000, Malvern Instruments GmbH, Herrenberg, Germany. Out of three measurements at 23 °C the average was taken using the same parameters as described for the Nicomp to determine the mean diameters.

### 2.3.3. Cryo-TEM

For comparison some selected liposome dispersions were additionally characterised regarding size and lamellarity by Cryo-transmission electron microscopy using a Zeiss CEM 902, Carl Zeiss, Oberkochen, Germany.

## 2.4. Encapsulation efficiency

Calcein was chosen as hydrophilic marker. It is widely used (Jousma et al., 1987; MacDonald et al., 1991; Grit and Crommelin, 1992) to determine encapsulation efficiency. Encapsulation efficiency (EE) was determined by equilibrium dialysis (ED) as described before (Schneider et al., 1995) using a Dianorm system with Diachema dialysis membranes (cut-off 10 000), Dianorm, Munich, Germany. The diluted liposome dispersion (1:100) was dialysed against a buffer solution for 2 h. Calcein was measured fluorimetrically (Kontron SFM 25, Kontron AG, Zurich, Switzerland, excitation at 489 nm, emission at 520 nm) against a calibration curve. To determine the calcein con-

centration in the liposomes they had to be destroyed with sodium cholate (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at a final concentration of 40-mM (Christine Tardi, personal communication). A potential interference of Na cholate with the calcein fluoresecence has been ruled out elsewhere (Tardi, 1999)

### 2.5. Lipid assays (HPLC)

The liposomes were dissolved in methanol before HPLC analysis. The employed columns were obtained from VDS optilab, Montabaur, Germany.

SPC and Lyso-SPC: a Lichrosorb $NH_2$ column was used with acetonitrile, methanol, 0.8% phosphoric acid in a ratio of 10:10:1 (v/v/v) as mobile phase. Flow rate: 1.5 ml/min, temperature: 20 °C, UV detection at 207 nm.

Cholesterol: an ODS Hypersil 5 μm column was used with methanol as the mobile. Flow rate: 1.0 ml/min, temperature: 30 °C, UV detection at 207 nm.

SPG: a Spherisorb $NH_2$, 5 μm column was used with acetonitrile, methanol, 0.1 M ammonium acetate buffer pH 4.8 in a ratio of 52:32:16 (v/v/v) as mobile phase. Flow rate: 1.0 ml/min, temperature: 20 °C, evaporative light scattering detector (Varex MKIII ELSD, Alltech Association, Inc., Deerfield, IL, USA), temperature: 75 °C.

### 2.6. Lamellarity

The lamellarity of the liposomes was determined by $^{31}$P-NMR measurements with a Bruker® Cryomagnet AC 300 or AC 400, Spectrospin, Fällanden, Switzerland. Liposomes were diluted (1:1) in buffer and $D_2O$ and $Pr(NO_3)_3$ (10 mM final concentration) were added (Fröhlich et al., 2001).

### 2.7. Flow rate and dead volume

The flow rate was determined by weighing the amount of liposome dispersion arriving at the outlet of the machine during one extrusion cycle and determining the time needed for that by a stop watch. The dead volume was determined by processing the liposome dispersion through the filter until the apparatus runs dry and afterwards flushing the apparatus five times with 100 ml of buffer each. The total amount of calcein recovered during flushing was quantified fluorimetrically (see above) and used to calculate the amount of product remaining in the machine after extrusion.

### 2.8. Contamination with metal traces

Aliquots of the liposome dispersions extruded by the continuous extruder were analysed for traces of iron, chromium, nickel and copper by atomic absorption spectroscopy (AAS) upon dilution with 0.1 M nitric acid (1:100). The instrument was an electrothermal Perkin Elmer Simaa 6000 equipped with graphite tube, run at a temperature program of 150, 1200 and 2000°C for drying, decomposition and atomisation, respectively. Quantitation was done against standard solutions of the four elements (mix of single element standards diluted to a range of 0.3–100 ppm) using home-made software for linear regression (Vespa).

## 3. Results

In order to compare the discontinuous to the continuous extruder, liposomes were prepared starting from the same raw dispersion and using identical process parameters (as, e.g. pore sizes and number of cycles). Liposome characteristics such as particle size, lamellarity and encapsulation efficiency were determined. In addition to this, the dispersions were assayed for loss of lipid and contamination with metal traces during extrusion. Dead volume and product flow of the devices were also measured.

### 3.1. Liposome size

For liposome size analysis, various approaches were compared: (1) PCS and Cryo-TEM; (2) alternative PCS instruments, namely Nicomp 370 and Malvern Zetasizer; and (3) alternative distribution fit modes, gaussian versus Nicomp-fit and volume-based versus number-based.

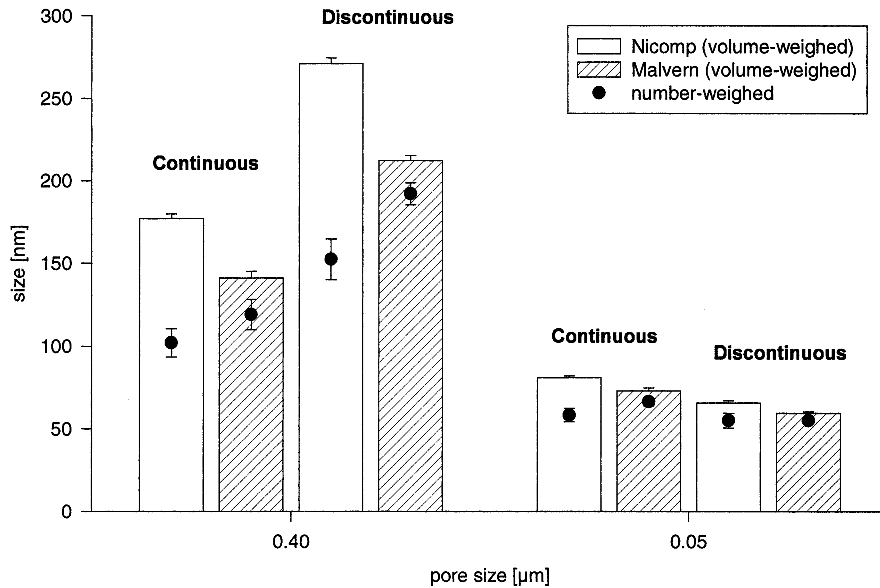


Fig. 2. Mean diameters of liposomes made by continuous (CE) and discontinuous (DE) extruder. Liposomes extruded down to final filter pore sizes of 0.4 and 0.05 μm respectively; sizes measured both with Nicomp and Malvern apparatus and using both volume- and number-based gaussian analysis (error bars denote S.D., $n = 3$).

Comparison of alternative ways of size analysis: the size distribution of both, continuously and discontinuously extruded liposomes at selected pore sizes (0.4 and 0.05 μm), was analysed with both a Nicomp and a Malvern instrument Fig. 2 shows the volume-based as well as the number-based mean diameters obtained by the two instruments in the gaussian-fit mode. In addition the smallest (0.05 μm pore-size) liposome dispersions were visualised using cryo-transmission electron microscopy. Representative electron micrographs are given in Figs. 3 and 4. Size distributions were determined on large-scale prints of a number of such micrographs. The results are given in Fig. 5. Both products showed unimodal, relatively narrow size distributions in the range of 20–100 nm. The distributions were asymmetric, steep to the lower end and with a tail towards bigger particle sizes. For both production products, Cryo-TEM resulted in mean particle diameters of 40–45 nm whereas the PCS revealed mean diameters of 53–58 nm. With the Nicomp instrument monomodal (gaussian) or, alternatively, bi- to oligomodal (Nicomp) fits can be selected. The software, however, recommended using the monomodal fit for all our preparations. By overriding the autoselection function, the effect of the chosen model on the calculated mean particle size and variability (gaussian fit) or on peak diameters and ratio of the sub-population peaks (Nicomp-fit) was studied. The results are shown in Table 1, both for CE and DE after extrusion through 5, 1 and 0.4 μm filters.

Comparison of the two extrusion devices: mean liposome sizes were measured after every extrusion step for DE and CE by using the Nicomp 370 in the gaussian fit-mode in parallel. In Fig. 6, both the volume-based and number-based mean diameters are given in comparison. The symbols and error bars represent mean and standard deviation of three batches, consecutively prepared under identical conditions. The exact figures for the filter stages 5, 1 and 0.4 μm are given in Table 1. Upon filtration through 0.1-μm-pore size CE resulted in a mean diameter of 98 nm and DE in a diameter of 112 nm. At the final pore size (0.05 μm), DE-liposomes had a mean diameter of 66 nm and CE-liposomes of 81 nm.

Table 1
Mean diameters of extruded liposomes obtained by PCS (Nicomp)

| | | Volume Gauss mean diameter (nm) coefficient variation | Volume Nicomp mean diameter (nm) (% amount) | | Number Gauss mean diameter (nm) | Number Nicomp mean diameter (nm) (% amount) | |
|---|---|---|---|---|---|---|---|
| | | | 1st peak | 2nd peak | | 1st peak | 2nd peak |
| CE | Batch 1 | 181.4 | 91.8 | 215.5 | 110.1 | 87.5 | 205.8 |
| | | 36.8% | (20%) | (80%) | | (76%) | (24%) |
| | Batch 2 | 182.3 | 74.7 | 212.5 | 101.0 | 71.6 | 203.2 |
| | | 39.5% | (16%) | (84%) | | (81%) | (19%) |
| | Batch 3 | 196.8 | 65.5 | 207.2 | 115.2 | 60.8 | 191.4 |
| | | 38.0% | (11%) | (89%) | | (79%) | (21%) |
| DE | Batch 1 | 276.1 | 102.5 | 311.7 | 150.8 | 98.0 | 289.3 |
| | | 41.5% | (10%) | (90%) | | (76%) | (24%) |
| | Batch 2 | 280.8 | 129.2 | 367.9 | 149.6 | 133.1 | 380.5 |
| | | 44.3% | (11%) | (89%) | | (76%) | (24%) |
| | Batch 3 | 277.1 | 100.5 | 311.0 | 156.0 | 97.3 | 290.0 |
| | | 40.5% | (10%) | (90%) | | (75%) | (25%) |

Liposomes extruded down to final pore size of 0.4 μm using continuous extruder (CE) or discontinuous extruder (DE), respectively. Sizes measured by Nicomp 370 using different fit modes (gaussian or Nicomp) and distribution types (volume or number-based size distribution).

### 3.2. Encapsulation efficiency

After each extrusion step encapsulation efficiency (EE) was measured.

In general, EE decreased with decreasing liposome sizes (Fig. 7). As the decrease in liposome sizes was more pronounced with DE compared to CE, there was also a more obvious reduction in the resulting EE.

### 3.3. Lamellarity

Lamellarity of liposomes prepared by DE or CE was studied as a function of filter pore size by $^{31}$P-NMR. Unextruded liposomes were too multilamellar to allow measurement (data not shown). Lamellarity was found to decrease with decreasing filter pore size yielding almost identical values for both extrusion methods at the different filtration

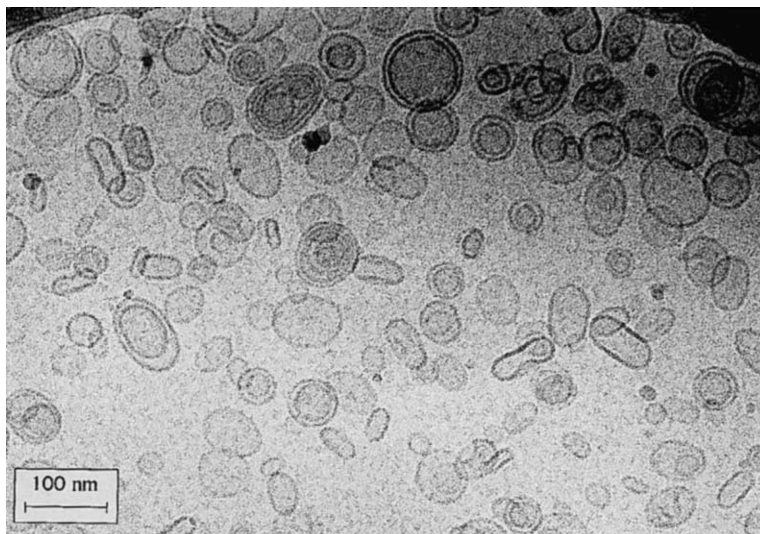


Fig. 3. Cryo-TEM micrograph of liposomes extruded by continuous extruder (CE). Liposome dispersion extruded down to final pore size of 0.05 μm.

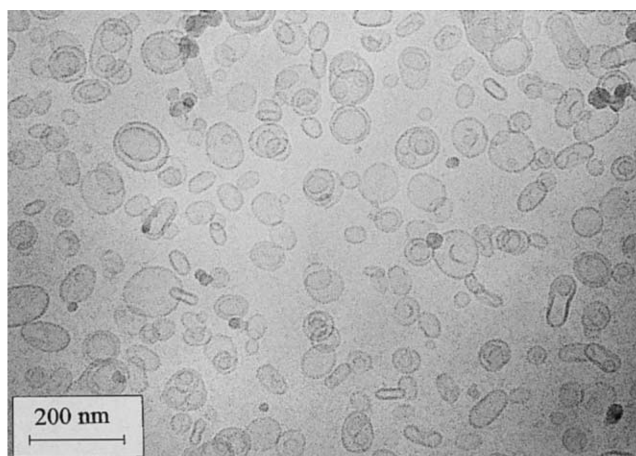


Fig. 4. Cryo-TEM micrograph of liposomes extruded by discontinuous extruder (DE). Liposome dispersion extruded down to final pore size of 0.05 μm.

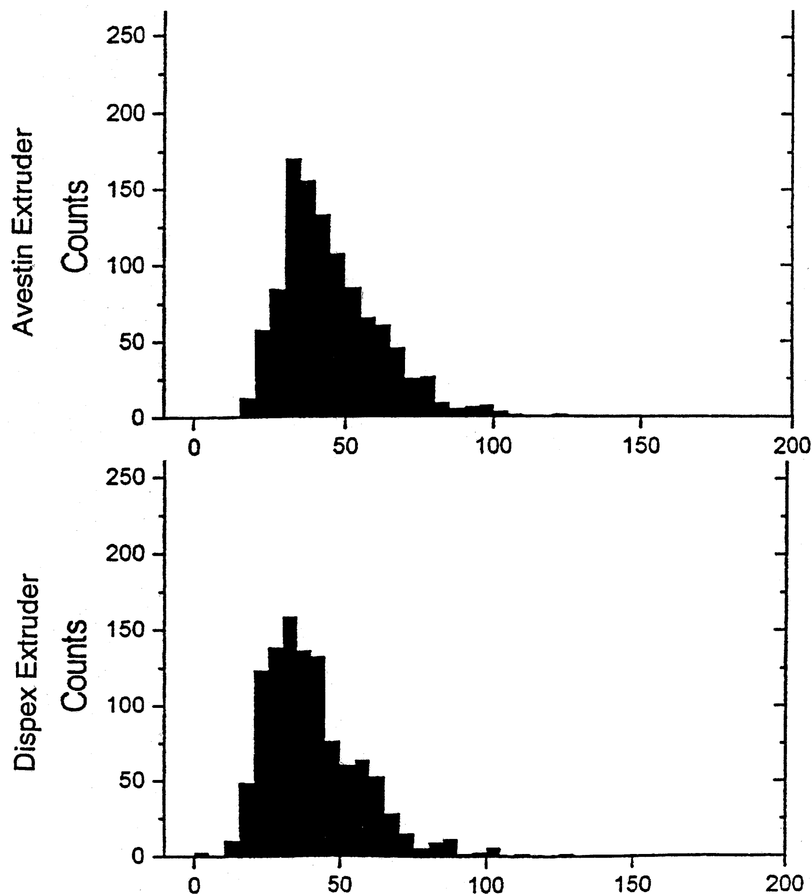


Fig. 5. Comparison of size distributions of DE and CE liposomes by cryo-TEM. Liposome dispersions extruded down to final pore sizes of 0.05 μm each.

stages (Table 2). The lamellarity value after 0.05-μm extrusion indicated unilamellarity of the respective preparations.

A typical NMR spectrum is given in Fig. 7.

### 3.4. Lipid assays

There are reports in the literature on lipid loss during extrusion (Jousma et al., 1987). Therefore, the employed lipids were quantified before and after extrusion down to pore sizes of 0.05 μm. Lipid analysis by HPLC detected no loss of lipids during liposome preparation, as shown in Table 3. There was a slight tendency towards up-concentration during the three freeze-thaw-cycles. The figures indicate no specific lipid loss but the amount of lyso-SPC was found slightly increased.

### 3.5. Process parameters

In order to compare the two extrusion devices process parameters such as flow rates and maximum achievable pressure were determined as well as the abrasion of metal particles from the devices during liposome production.

There was a marked difference in flow rates between CE and DE (Table 4). Even for big filter pore sizes of 5.0, 1.0 and 0.4 μm, where no pressure could be measured above the membranes, flow rates were about threefold higher in CE. When using smaller pore sizes, the flow rates became even more divergent. It has to be mentioned that 52 bar was the technical limit for the DE, whereas CE was performed at pressures as high as 75 bar (technical limit 120 bar). In most

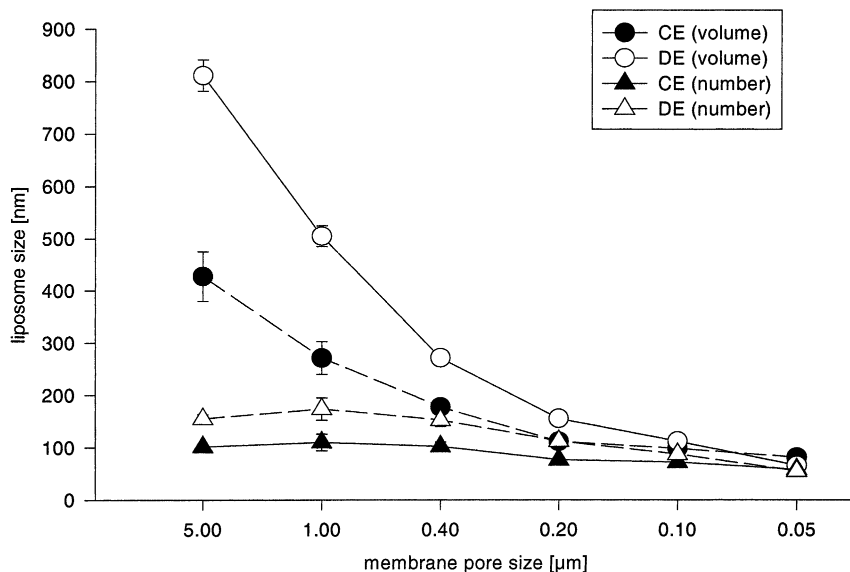


Fig. 6. Mean diameters of liposomes extruded by continuous (CE) and discontinuous (DE) extruder as a function of decreasing filter pore sizes. Sizes of CE and DE liposomes as obtained by Nicomp PCS analysis and expressed both as volume and number-based mean sizes (error bars denote S.D., $n = 3$).

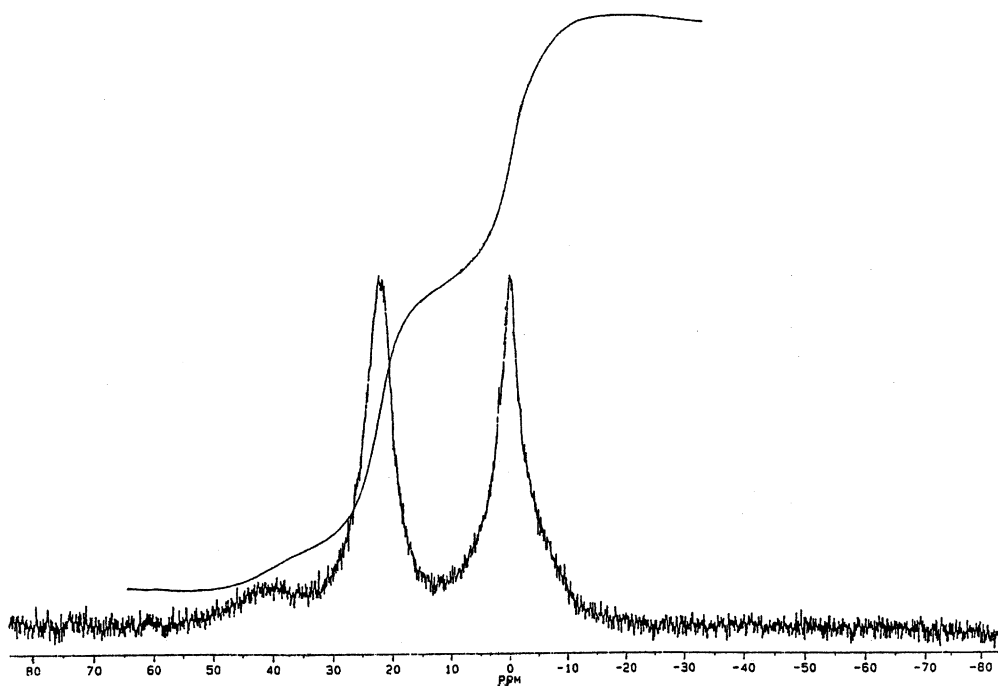


Fig. 7. $^{31}$P-NMR spectrum of CE liposomes extruded down to final pore size of 0.05 μm. Spectrum taken upon addition of shift reagent ($Pr(NO_3)_3$).

Table 2
Mean lamellarities of extruded liposomes measured with $^{31}$P-NMR

| Pore size (μm) | Continuous extruder | Discontinuous extruder |
|---|---|---|
| 1.0 | 1.41 | 1.33 |
| 0.4 | 1.23 | 1.28 |
| 0.2 | 1.08 | 1.09 |
| 0.05 | 1.03 | 0.99 |

Liposomes prepared in 20 mM Tris/HCl buffer pH 7.4 and extruded step-wise using the continuous and discontinuous machine respectively. Lamellarities determined from the ratios of the original to the shifted peaks upon addition of shift reagent ($Pr(NO_3)_3$).

cases flow rates decreased with decreasing pore sizes. Interestingly, no difference in flow rate was found between 0.2- and 0.1-μm pore size in DE, whereas in CE the flow rate, however, increased. This could be due to the nominal thickness of the polycarbonate membranes decreasing from 10 (0.2 μm) to 6 μm (0.1 μm) although porosity ($3 \times 10^8$ pores/cm$^2$) was constant. The shorter way through the 0.1-μm pores apparently allowed higher flow rates.

In the LiposoFast™ the polycarbonate filters are placed on a polypropylene drain disk. This is expected to affect the flow rate.

Atomic absorption spectroscopy (AAS) analysis on iron, chromium, nickel and copper was performed in order to detect any metal abrasion from the extruder. Since the DE comprises no moving parts (e.g. pump piston) it was assumed that no abrasion could occur.

For CE liposomes the only significant metal trace contamination that could be detected was an elevated amount of chromium (Table 5). The AAS analysis was performed after extrusion but

Table 3
Recovery of the employed lipids after freeze-thaw and extrusion

| Lipid | Calculated amount (mg/ml) | Before extrusion (mg/ml) | Continuous extruder (mg/ml) ($n = 3$) | Discontinuous extruder (mg/ml) ($n = 3$) |
|---|---|---|---|---|
| SPC | 110.8 | 115.1 | $114.0 \pm 0.19$ | $116.0 \pm 0.87$ |
| CH | 25.7 | 26.7 | $26.0 \pm 0.25$ | $26.5 \pm 0.48$ |
| SPG | 13.5 | 13.5 | $13.4 \pm 0.24$ | $13.6 \pm 0.33$ |
| Lyso-SPC | 0.9[a] | 2.0 | $2.4 \pm 0.05$ | $3.0 \pm 0.14$ |

Liposomes prepared by thin-film hydration, freeze-thaw and subsequent step-wise extrusion down to final pore size of 0.05 μm using the continuous and discontinuous machine, respectively. Concentrations of the lipid components (SPC, CH and SPG) as well as the main degradation product (Lyso-SPC) measured by HPLC.

[a] Employed SPC contains 0.9% lyso-lecithin (certificate of analysis).

Table 4
Flow rate and extrusion pressure during extrusion of liposome dispersions

| Pore size (μm) | Continuous extrusion | | Discontinuous extrusion | |
|---|---|---|---|---|
| | Flow rate (ml/min) | Pressure (bar) | Flow rate (ml/min) | Pressure (bar) |
| 5.0 | 1079 | 0 | 313 | 0 |
| 1.0 | 1068 | 0 | 329 | 0 |
| 0.4 | 896 | 25 | 273 | 4 |
| 0.2 | 649 | 75 | 130 | 25 |
| 0.1 | 928 | 65 | 123 | 40 |
| 0.05 | 616 | 75 | 21 | 52 |

Liposome dispersions extruded using the continuous and discontinuous machine respectively. Flow rates calculated from the measured passage times of defined volumes of product. Extrusion pressure represents maximum observed pressure above the membrane during extrusion.

Table 5
Traces of metals in continuously extruded liposomes as determined by AAS

| Metal | Blind | CE 0.4 μm ppm[a] (95% CI[b]) | CE 0.1 μm ppm[a] (95% CI[b]) |
|---|---|---|---|
| Fe | <1 | 1 (0.9–1.6) | 2 (1.2–2.0) |
| Cr | <0.02 | 0.16 (0.13–0.20) | 0.26 (0.22–0.29) |
| Ni | <0.5 | <0.5 | <0.5 |
| Cu | <0.5 | <0.5 | <0.5 |

Liposomes continuously extruded down to final pore sizes of 0.4 and 0.1 μm, respectively. Metal traces determined in liposome dispersions by AAS as an indicator for abrasion of metal particles.

[a] ppm = mg/kg.

[b] 95% CI = 95% confidence.

before sterile-filtration through cellulose acetate membranes. The amount of chromium and iron in the suspension increased with the number of extrusion steps (final pore size $0.1 > 0.4$ μm).

Dead volume in CE was 5.6 ml, in DE 3.4 ml. This difference is regarded small with respect to the huge difference in product-contacting surface areas of the two machines (DE much smaller).

Maximum extrusion pressure was 54 bar for DE and 120 bar for CE. This allowed a much faster extrusion process using the CE. For example production of a 200-ml batch in the discontinuous mode, i.e. collecting each fraction before next extrusion cycle required a process time of 50 min. With DE 230 min were needed. Process times for CE would be even shorter if extrusion would be performed in a continuous mode.

## 4. Discussion

In order to compare size analysis techniques selected liposome dispersions were analysed using two different PCS analysers (Nicomp 370 and Malvern Zetasizer) and cryo electron microscopy in parallel. There was almost no difference found for smaller liposomes (final pore size 0.05 μm) when comparing the two PCS instruments no matter if volume- or number-based size distributions were used (Fig. 2). In contrast, for larger particles (extruded through 0.4 μm), the following differences were detected between the two instruments: In the volume-based mode the Nicomp reported larger mean diameters for both samples (CE and DE) than the Malvern, whereas in the number based mode it was the other way round. Apparently, the Nicomp is weighting the bigger particles more than the Malvern. When the Nicomp-fit was used, two sub-populations were obtained (Table 1), where the larger vesicles always accounted for approximately 80–90% of the total population (volume-based). When analysing liposome dispersions after extrusion through 0.05 μm filter pore size, the mean diameters obtained by all three techniques (Nicomp, Malvern and Cryo-TEM) were found to be in good agreement with each other, regardless which fit was used. In summary, it is obvious that the particle size analysis technique itself may have a tremendous influence on the results obtained. This indicates that it would be inappropriate to directly compare vesicle sizes reported in different studies with each other, except if the equivalence of the analytical approaches can be demonstrated.

Comparison of the two extrusion devices was done using the Nicomp in the gaussian mode because this approach appeared most sensitive to bigger liposomes, which is relevant for parenterally applicable liposome dispersions. In general, in the course of the filter extrusion, a decrease in particle size with decreasing pore sizes was observed. Size reduction mechanism was suggested to include the rupturing of vesicles and spontaneous rearrangement after membrane passage resulting in the formation of smaller and less lamellar liposomes (Lesieur et al., 1991). When using relatively big filter pores (0.4 μm and above) the resulting mean diameters of the vesicles were smaller than the pore size. Smaller pore sizes (0.2 μm and below) resulted in vesicle diameters slightly bigger than the nominal pore size. This can be attributed to elastic deformation of the liposome spheres to ellipsoid shape (Lesieur et al., 1991). These elastically deformed ellipsoid particles pass easier through the pores (Olson et al., 1979). The existence of such elliptic liposomes after extrusion has been demonstrated earlier by Cryo-TEM (Schneider, 1994).

When comparing liposomes sizes at different filtration stages obtained by the two extrusion techniques, distinct differences were detected (Fig. 6): CE liposomes at all stages had smaller mean

diameters than DE liposomes except for the final pore size (0.05 μm), where no significant differences were found. The differences in size was much more pronounced for the first steps (5.0, 1.0 and 0.4 μm) and levelled out during subsequent extrusion. The size difference between CE and DE was seen irrespective of the employed size analysis mode but was most obvious in the volume-based fit. In the Nicomp-fit, both sub-populations (Table 1) appeared to have larger diameters, whereas the ratio of the two sub-populations appeared quite constant.

This is not in congruence with the theory that preferentially large particles undergo size reduction during filter extrusion. The size difference between CE and DE liposomes was attributed to the difference in flow rate and also partly in pressure drop over the membrane. It is assumed that all of the above described effects which are made responsible for particle size reduction during filter extrusion would to a larger extent occur with higher flow rates and bigger pressure differences.

Encapsulation efficiencies were determined for filter pore sizes of 0.4 μm and below (Fig. 8). In general, encapsulation efficiencies between 15 and 50% were found which are much higher than most literature values. Reported EEs for fluorescence markers were about 2–6% with a liposome size of approx. 70 nm (MacDonald et al., 1991), about 4.5% with a liposome size of approx. 108 nm (Elorza et al., 1993) or about 8% (vesicles approx. 140 nm) and about 13.5% (vesicles approx. 280 nm) (Jousma et al., 1987). This is attributed to the much lower lipid concentrations of < 50 mg/ml in these studies compared to the 150 mg/ml used here. Schneider has demonstrated the dependence of encapsulation efficiency of extruded liposomes on lipid concentration (Schneider et al., 1995).

The EEs were found to correlate quite well with liposome sizes. During stepwise extrusion, both liposome size and EE decreased. As CE for almost all filter pore sizes resulted in smaller liposomes than DE, the corresponding EE values were also found to be lower with CE than DE liposomes. Only at 0.05 μm pore size, CE liposomes were bigger (81 nm) and a slightly higher EE (20%) were determined compared to liposome sizes of 66 nm and EE of 15% with DE.

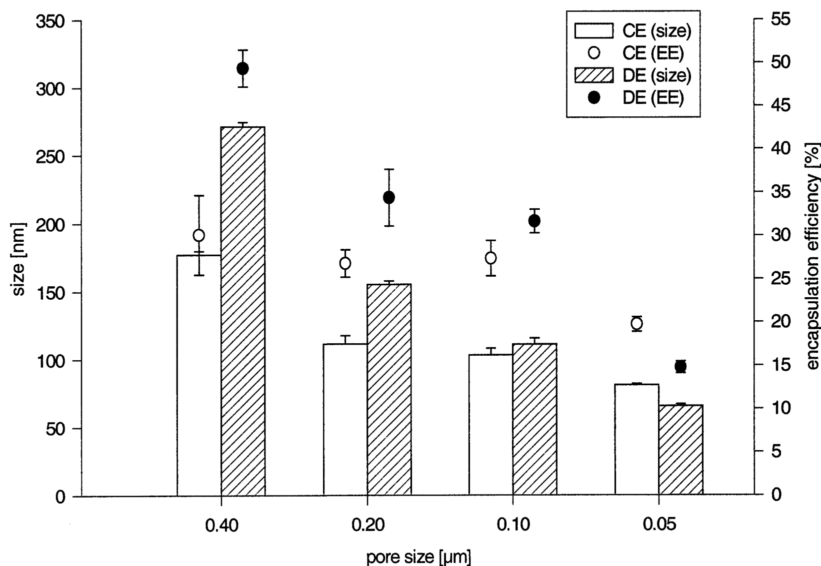


Fig. 8. Comparison of mean liposome sizes and encapsulation efficiencies for liposomes prepared by continuous (CE) and discontinuous (DE) extruder. The mean liposome sizes represent volume based diameters as obtained by Nicomp PCS analysis. EE was measured by quantifying the calcein content (error bars denote S.D., $n = 3$).

Lipid recovery (Table 3) measured by HPLC amounted to 100% or above. Apparently no lipid loss occurred during filter extrusion, no matter which extruder was used. This is in contradiction to studies by Jousma et al. (1987) who had determined a loss of lecithin. This may be due to the lower extrusion pressures employed in their study. Lipid concentrations of more than 100% of the theoretically calculated lipid amounts measured in the present study were attributed to a minor loss of water during the thawing process at 70 °C (evaporation). The amount of lyso-SPC was slightly increased. This phenomenon is commonly observed with aqueous lipid dispersions and not regarded specific for filter extrusion.

In conclusion: (1) the main difference between Dispex extruder (CE) and Avestin extruder (DE) was higher flow rates and pressure for CE which resulted in smaller liposomes and reduced encapsulation efficiencies at intermediate filter pore sizes whereas the final products after extrusion through 0.05 μm were similar. The higher flow rates result in much shorter processing times. (2) Major differences between size analysis techniques (Nicomp PCS, Malvern PCS and Cryo-TEM) were observed with hererogeneous dispersions of bigger liposomes, whereas for homogeneous dispersions of small liposomes almost no difference was observed. (3) DE was employed at its technical limits here. For CE it would be interesting to employ higher lipid concentrations and check if it is feasable to obtain liposomes with even higher EE.

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